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Signed P. Mahoney

Dated 5 October 1999.

For official use



Your Reference: B45160

16 OCT 1998

**9822714.3**

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# The Patent Office

## Request for grant of a Patent

Form 1/77 **Patents Act 1977**

**❶ Title of invention**

1 Please give the title of the invention: VACCINES

**❷ Applicant's details**

☐ **First or only applicant**

2a If you are applying as a corporate body please give:
Corporate Name: SmithKline Beecham ~~S.a.~~ Biologicals S.A.

Country *(and State of incorporation, if appropriate)*: Belgium

2b If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

2c **In all cases,** please given the following details:

Address: Rue de l'Institut 89, B-1330, Rixensart

UK postcode *(if applicable)*

Country: Belgium

ADP number *(if known)*: ~~5800974002~~ ~~7533243001~~ 06510956001

**2d, 2e and 2f:** *If there are further applicants please provide details on a separate sheet of paper*

☐ **Second applicant** ***(if any)***

2d If you are applying as a corporate body please give:
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Surname:

Forenames:

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ADP number *(if known)*

③ *An address for service in the United Kingdom must be supplied*

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③ **Address for service details**

3a Have you appointed an agent to deal with your application?
Yes ☒ No ☐ ➲ ***go to 3b***

⇩

***please give details below***

Agent's name MARCUS J W DALTON

Agent's address SmithKline Beecham
Corporate Intellectual Property
New Horizons Court
Great West Road
Brentford, Middlesex

Postcode TW8 9EP
Agent's ADP number 580074002

**3b:** *If you have appointed an agent, all correspondence concerning your application will be sent to the agent's United Kingdom address.*

3b If you have not appointed an agent please give a name and address in the United Kingdom to which all correspondence will be sent:

Name

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Daytime telephone *(if available)*

## ❹ Reference number

4. Agent's or applicant's reference number *(if applicable)* B45160/MJWD

## ❺ Claiming an earlier application date

5. Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

*Please mark correct box*

Yes ☐ No ☒ ➲ *go to 6*

*please give details below*

☐ number of earlier application or patent number

☐ filing date *(day month year)*

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15(4) (Divisional) ☐ 8(3) ☐ 12(6) ☐ 37(4) ☐

## ❻ Declaration of priority

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❻ *If you are declaring priority from a PCT Application please enter 'PCT' as the country and enter the country code (for example, GB) as part of the application number*

*Please give the date in all number format, for example, 31/05/90 for 31 May 1990*

6. If you are declaring priority from previous application(s), please give:

| Country of Filing | Priority application number *(if known)* | Filing Date *(day, month, year)* |
|---|---|---|
| | | |

⑦

- any applicant is not an inventor
- there is an inventor who is not
- an applicant, or
- any applicant is a corporate body.

## ⑦ Inventorship

7. Are you (the applicant or applicants) the sole inventor or the joint inventors?

*Please mark correct box*

Yes ☐ No ☒ *A Statement of Inventorship on Patents form 7/77 will need to be filed (see Rule ○.).*

⑧ *Please supply duplicates of claim(s), abstract, description and drawings).*

## ⑧ Checklist

8a Please fill in the number of sheets for each of the following types of document contained in this application

| | |
|---|---|
| Continuation sheets for this Patents Form 1/77 | – |
| Claim(s) | |
| Description | 15 |
| Abstract | |
| Drawing(s) | |

8b Which of the following documents also accompanies the application?

*Please mark correct box(es)*

| | |
|---|---|
| Priority documents *(please state how many)* | – |
| Translation(s) of Priority documents *(please state how many)* | – |
| Patents Form 7/77 - Statement of Inventorship and Right to Grant | – |
| Patents Form 9/77 - Preliminary Examination Report | – |
| Patents Form 10/77 - Request for Substantive Examination | – |

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## ⑨ Request

I/We request the grant of a patent on the basis of this application.

*Please sign here* ✍

Signed Marcus J W Dalton Date: 16/10/98 *(day month year)*

MARCUS J W DALTON

Chartered Patent Attorney

Attorney for the Applicant

*A completed fee sheet should preferably accompany the fee.*

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# Vaccines

The present invention relates to new vaccine compositions, processes for their manufacture and their use in medicine. In particular, the present invention relates to improved Hepatitis A vaccines adjuvanted with a potent immunostimulator, preferably such as monophosphoryl lipid A or derivative thereof. The invention also relates to combination vaccines in which the Hepatitis A vaccine is a component.

Hepatitis A vaccines are known. For example the vaccine Havrix (Trade Mark), from SmithKline Beecham Biologicals can be used to prevent hepatitis A infections and is also formulated with aluminium hydroxide as adjuvant. This vaccine is produced according to a modified procedure (no CS CL gradient) of Andre et al. It comprises an attenuated strain of the HM-175 Hepatitis A virus inactivated with formol (formaldehyde); see Andre et al [Prog Med. Virol. 1990, vol 37; -p72-95]. The vaccine Twinrix (Trade Mark) which is a combination of the above hepatitis A and hepatitis B antigens may be used to protect against Hepatitis A and Hepatitis B simultaneously.

International patent application WO93/19780 (SmithKline Beecham Biologicals s.a.) discloses, inter alia, a Hepatitis A vaccine adjuvanted with 3D-MPL.

European patent 0 339 667 (Chemo Sero) describes the general concept of combining a hepatitis A antigen and a hepatitis B antigen to make a combination vaccine. In that specification it is stated that the adjuvant which is used is not critical: it must only be capable of enhancing the immune activity to a desired extent and not cause any side effects. It is stated that aluminium gel may be used, in particular aluminium hydroxide gel and aluminium phosphate gel.

It has now been found that traditional processes for producing and purifying inactivated virus for hepatitis A vaccines leave a small residue of contaminants from the host cells upon which the hepatitis A vaccine was grown. Such host cell contaminants are at a level that provide no concern when the vaccine is adjuvanted with aluminium salts, but when adjuvanted with strong immunostimulants it is possible that a vaccinee may raise an adverse immune response to the host contaminants.

Accordingly there is a need for a method of manufacture which removes substantially all traces of such host cell proteins.

Accordingly in one embodiment of the invention there is a process for the production of inactivated Hepatitis A virus substantial free of host cell contamination, the process comprising:

a) a culturing Hepatitis A vaccine and harvesting said hepatitis A preparation

b) treating said hepatitis A preparation with a protease, thereafter

c) separating intact virus from small protease digested protein

d) inactivating said virus.

Preferably the Hepatitis A virus is derived from HM-175 strain.

By substantially free of host cell contamination it means that less than 10%, preferably less than 8% more preferably less than 5% protein can be detected by method SDS PAGE and silver staining. As determined by slot blot method one dose of HAV in the vaccine preferably contains less than 10 ng of host cell proteins.

Preferably the protease used is trypsin. Other proteases that may be utilised included pronase, papain, and pepsin.

The protease treatment is preferably carried out at above room temperature, e.g. at about 37°C for about 2 hrs.

The separation of the intact virus from the protease and the digested components can be achieved by permeation chromatography.

Alternatively the protease may be separated by any chromalographic method that separates on the basis of size, for example ultra filtration.

The product can then be further purified, by other steps to remove other contaminants. For example by subjecting the product to ion-exchange chromatography to remove any nucleic acid contamination.

Accordingly, in one embodiment of the present invention there is provided an inactivated Hepatisis A virus substantially free of contaminating host proteins.

The inactivated hepatitis A virus vaccine may then be formulated into a vaccine. Thus the invention provides a Hepatitis A vaccine comprising an inactivated hepatitis A virus substantially free of host cell contaminants.

Such a vaccine may advantageously include a suitable adjuvant. Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

Advantageously, the highly purified hepatitis A virus may be formulated with strong adjuvant systems. Thus in the formulation of the invention, it is preferred that the adjuvant composition induces an immune response comprising TH1 aspects. Suitable adjuvant systems include, for example a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salts.

An enhanced system involves the combination of monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO 96/33739.

A particularly potent adjuvant formulation involving QS21 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210.

Other known adjuvants which may be included are CpG containing oligonucleotides.

Accordingly in a preferred embodiment of the present invention there is provided a vaccine comprising a virus of the present invention, adjuvanted with a monophosphoryl lipid A or derivative thereof.

Preferably the vaccine additionally comprises a saponin, more preferably QS21.

Preferably the formulation additional comprises an oil in water emulsion and tocopherol. The present invention also provides a method for producing a vaccine formulation comprising mixing a protein of the present invention together with a pharmaceutically acceptable excipient, such as 3D-MPL.

The purified virus of the invention may advantageously be combined with other antigens so that it is effective in the prophylaxis or treatment of other diseases in addition to hepatitis A infections. A preferred combination involves a combination containing a hepatitis B antigen.

The preparation of Hepatitis B surface antigen (HBsAg) is well documented. See, for example, Harford et al in **Develop. Biol. Standard** 54, page 125 (1983), Gregg et al in **Biotechnology**, 5, page 479 (1987), EP-A-0 226 846, EP-A-0 299 108 and references therein.

As used herein the expression 'Hepatitis B surface antigen' or 'HBsAg' includes any HBsAg antigen or fragment thereof displaying the antigenicity of HBV surface antigen. It will be understood that in addition to the 226 amino acid sequence of the HBsAg S antigen (see Tiollais et al, Nature, 317, 489 (1985) and references therein) HBsAg as herein described may, if desired, contain all or part of a pre-S sequence as described in the above references and in EP-A- 0 278 940. In particular the HBsAg may comprise a polypeptide comprising an amino acid sequence comprising residues 12-52 followed by residues 133-145 followed by residues 175-400 of the L-protein of HBsAg relative to the open reading frame on a Hepatitis B virus of ad serotype (this polypeptide if referred to as L*; see EP 0 414 374). HBsAg within the scope of the invention may also include the preS1-preS2-S polypeptide described in EP 0 198 474 (Endotronics) or analogues thereof such as those described in EP 0 304 578 (McCormick and Jones). HBsAg as herein described can also refer to mutants, for example the 'escape mutant' described in WO 91/14703 or European Patent Application Publication Number 0 511 855 A1, especially HBsAg wherein the amino acid substitution at position 145 is to arginine from glycine.

Normally the HBsAg will be in particle form. The particles may comprise for example S protein alone or may be composite particles, for example (L*,S) where L* is as defined above and S denotes the S-protein of HBsAg. The said particles is advantageously in the form in which it is expressed in yeast.

The invention in a further aspect provides a vaccine formulation as described herein for use in medical therapy, particularly for use in the treatment of prophylaxis of hepatitis viral infections. In a preferred aspect the vaccine accordingly to the invention is a therapeutic vaccine useful for the treatment of ongoing hepatitis infections, more especially hepatitis A and/or hepatitis B infections in humans suffering therefrom.

In view of the surprisingly efficaceous results obtained, in a further preferred aspect the invention provides a vaccine composition for the treatment of prophylaxis of Hepatitis A and/or Hepatitis B infections.

Advantageously the hepatitis vaccine composition of the invention contains other antigens so that it is effective in the treatment or prophylaxis of one or more other bacterial, viral or fungal infections.

Accordingly the hepatitis vaccine formulation according to the invention preferably contains at least one other component selected from non-hepatitis antigens which are known in the art to afford protection against one or more of the following diseases:

diphtheria, tetanus, pertussis, Haemophilus influenzae b (Hib), and polio.

Preferably the vaccine according to the invention includes HBsAg as hereinabove defined.

Suitable components for use in such vaccines are already commercially available and details may be obtained from the World Health Organization. For example the IPV component may be the Salk inactivated polio vaccine. The pertussis vaccine may comprise whole cell or acellular product.

Advantageously the hepatitis or combination vaccine according to the invention is a paediatric vaccine.

The amount of antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending on which specific immunogens are employed. Generally it is expected that each dose will comprise 0.01 to 1.0μg protein/dose immunogen, most preferably from between .06 to .150μgprot/dose. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects may receive a boost in about 4 weeks.

In a further aspect of the present invention there is provided a method of manufacture of a vaccine effective in preventing or treating hepatitis infection, wherein the method comprises mixing the hepatitis antigen as defined herein with MPL or derivative thereof.

Using this method one or more additional components are preferably admixed with the inactivated Hepatitis A vaccine to provide a combination vaccine.

The following examples illustrate the invention and its advantages.

**Examples:**

Purification of Hepatitis A virus strain HM-175 was carried out according to the protocol shown in Figure 1.

Hepatitis A virus HM-175 was cultured on MRC5 cells (Andre et al supra) and the virus harvested in the presence of detergent - Tween- in order to lyse the cells and then after washing to remove serum contamination, filtered through a 0.22μm membrane and subjected to ultra filtration followed by gel permeation chromatography on a sepharose 4B-CL-FF prior to treatment with trypsin.

**TRYPSINATION**

The pool of fractions containing the HAV virus is treated with purified trypsin extracted from pig pancreas. The trypsin used is double crystallised and kept frozen before use. Before addition of trypsin the pool is prewarmed at 37°C under constant agitation. Trypsin is then added with a ratio of 440 IU per ml of pool, and the mixture is gently stirred for minimum 2H at 37°C (maximum 2.5H).

**CONCENTRATION**

After trypsin treatment the product is processed without delay at ambient temperatures on an ultrafiltration device in order to reduce the volume. The membrane used is regenerated cellulose with nominal cut-off of 30.000 Dalton, and up to maximum 8ml of trypsinated product per $cm^2$ of membrane, is processed at a transmembrane pressure: between 0.2 and 0.6 bar to achieve a concentration factor of between 8 and 12.

**2nd PERMEATION CHROMATOGRAPHY**

The aim of this step is to separate trypsin digested proteins from the unaffected HAV viruses. The separation gel used is Permeation Sepharose 4BFF. The virus is eluted at a smaller retention volume than the smaller protein fragments which are eluted with larger retention volume (closer to the total volume of the column).

Chromatography parameters are as follow:

Chromatographic medium: Sepharose 4B FF (from Pharmacia)

Injected volume: 1 to 5% of gel volume

Elution rate: 5 cm/h

Temperature: 10 to 16°C.

Pool of fractions: target 100 ng prot/ 720 Elisa units (+/- 10%)

**ION EXCHANGE**

The purpose of this purification step is to reduce the DNA content (originating from MRC5 cells).

This step is run according to the batch principle.

The pool from the previous chromatographic step is adjusted to 0.3M NaCl and then mixed with the ion exchange resin under mild agitation for 1H (maximum 1.5H) at room temperature.

After DNA fixation the gel is eliminated by filtration.

The unfixed HAV viruses suspension is then diluted to adjust the NaCl concentration to 150mM.

The final purified product is sterile filtered on 0.22$\mu$m filter.

Chromatographic parameters are as follow:

Load: 3% of gel compared to the volume of the pool (vol/vol).

Temperature: ambient

Inactivation was carried out as described in Andre et al, except that 100$\mu$g/ml of phenol was used. 250 $\mu$g/ml formol

**FORMALDEHYDE REDUCTION**

Within 48H after the end of the inactivation the product is diafiltered and concentrated in order to reduce the formaldehyde content and to be preadsorbed on aluminium hydroxide.

Before use the complete ultrafiltration device is sanitised with 0.1N NaOH for at least 30 minutes. The device is then thoroughly rinsed with diafiltration buffer and the membranes are then coated with a buffer containing amino acids (Travasol). Finally the device is rinsed with diafiltration buffer.

After diafiltration and concentration the final product is sterile filtered on 0.22$\mu$m filter.

**Example 1b**

In an alternative purification scheme, the trypinisation step is carried out between the ultrafiltration and first permeation chromatography steps. This means that the second permeation chromatography step can be eliminated leading to higher yields of the final product.

**Example 2**

**Characterisation:**

Samples of purified product was analysed by SDS PAGE 12.5 % acrylamide, 1% SDS in the stacking gel,migration for 15 h at 45-50 volts. Gel is stained with AgNO3 and the colour is allowed to develop for 10 to 20 min and compared with traditional HAV processes (Andre et al).

As can be seen from figure 3 and 4 subjecting the product to protease treatment a majority of high molecular weight contaminants are removed

**Example 3**

**HAV vaccine formulations**

**3.1 HAV - alum 3D-MPL**

The HAV particle of example 1 is first adsorbed on to aluminium hydroxide (superfos) followed by the addition of free 3D-MPL. A 0.5 ml dose 720 ELU of Hepatitis A virus particle / 0,025 mg A13+ ion and 50µg of 3D-MPL.

**3.2 HAV + Hbs Ag formulations**

The following formulations were made:

1. Hep B S Ag 20µg / $A1P0_4$ + HA 720 / $A1(OH)_3$
2. Hep B S Ag 20µ / $A1PO_4$ / 3D-MPL 50 µg + HA 1440 / $A1(OH)_3$
3. Hep B S Ag 20µg / $A1PO_4$ + 3D-MPL 50 Mg /A1 $(OH)_3$ + HA 720 / $A1(OH)_3$
4. Hep B S Ag 20µg / $A1PO_4$ + 3D-MPL 50µg / $A1PO_4$ + HA 720 $A1(OH)_3$
5. Hep B S Ag 20/µg / $AlPO_4$ / 3D-MPL 50µg + HA 720 / $Al(OH)_3$

In group 1 the individial antigens are adsorbed on to the aluminium salt 0.025mg A13+ion ($A1_3OH$ Superfos) for HA, 0.475 mg A13+ion (A1P04 Superfos type). In group 2 and 5, 50µg/dose of free 3D-MPL is addeded to

adsorbed Hepatitis S antigen to which the adsorbed hepatitis A component is added. In group 3 and 4, 3D-MPL is separately adsorbed on to the aluminium salt, and then the three adsorbed components are mixed together.

**Example 4 - Immunogenicity experiments**

**Balb/c mice**

Groups of 10 mice were immunised intramuscularly three times at 2 weeks interval with HAV/HBs formualtions (1/10 HD). Antibody response to Hbs were monitored by ELISA at 14 days post II and 14 days post III. The isotypic profile of the anti-HBs response was analysed at 14 days post II. Antibody response to HAV was monitored 14 days post III.

NMRI mice

Groups of 10 mice were immunised intraperitoneally once with HAV/HBs formulations (1/2 HD). Antibody response to Hbs and HAV were monitored by ELISA at 28 days post injection.

**Formulations**

| Group | Vaccine lot | Formulation |
|---|---|---|
| 1 | HAB112B6 | HBs 20µg / AlPO4 + HAV 720 / Al(OH)3 |
| 2 | DHAB713 | HBs 20µg / AlPO4 / MPL 50 + HAV 1440 / Al(OH)3 |
| 3 | DHAB717 | HBs 20µg / AlPO4 + MPL 50 / Al(OH)3 + HAV 720 / Al(OH) 3 |
| 4 | DHAB718 | HBs 20µg / AlPO4 + MPL 50 / AlPO4 + HAV 720 / Al(OH)3 |
| 5 | DHAB716 | HBs 20µg / AlPO4 / MPL 50 + HAV 720 / Al (OH) 3 |

**HAV Mouse Serology**

Quantitation of anti-Hepatitis A Virus antigen (HAV) antibody was performed using Enzygnost kit from Behring (ref: OQEC11). This assay is an ELISA based on the competitive test principle, run in one step and initially developed for human serology.

Two-fold dilution of mice sera (4 dilusions starting at 1/10) human anti-HAV reference (8 dilusions starting at 80 mIU/ml) and controls were performed in anti-HAV negative human sera. Mixtures of test/control samples (25µl), HAV

antigen solution (50µl) and anti-HAV mouse monoclonal conjugated with peroxydase (50µl of 1/41 dilution performed in conjugate buffer) were incubated on HAV pre-coated microplates for 2 hrs at 37°c. The plates were then washed and incubated for 30 min with a solution of TMB (100µl). The reaction was stopped with $H_2SO_4$ 0.5N and read at 450/620 nm.

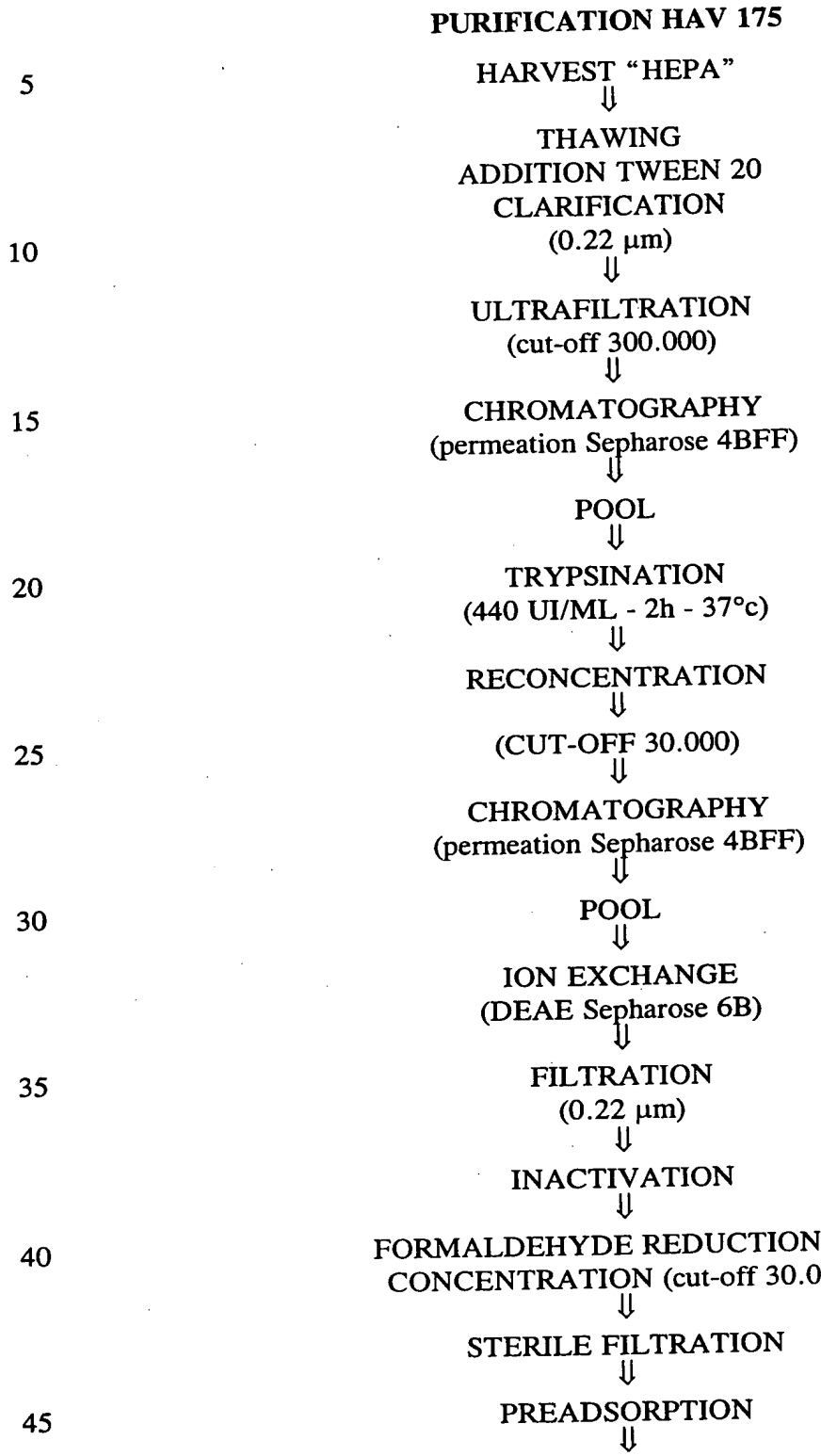
Anti-HAV antibody titers were calculated from the reference by SoftmaxPro (using a four parameters equation) and expressed in mlU/ml.

**Results**

The results are shown in figure 2.

In figure 2a results demonstrate that formulations containing MPL induce significantly higher antibody responses to the hepatitis A component than the aluminum salt group alone. Similarly the results shown in figure 2b demonstrate that MPL containing formulations induce higher antibody titres to HbsAg.

**Figure 1**


PURIFICATION HAV 175
HARVEST "HEPA"
THAWING
ADDITION TWEEN 20
CLARIFICATION
(0.22 µm)
ULTRAFILTRATION
(cut-off 300.000)
CHROMATOGRAPHY
(permeation Sepharose 4BFF)
POOL
TRYPSINATION
(440 UI/ML - 2h - 37°c)
RECONCENTRATION
(CUT-OFF 30.000)
CHROMATOGRAPHY
(permeation Sepharose 4BFF)
POOL
ION EXCHANGE
(DEAE Sepharose 6B)
FILTRATION
(0.22 µm)
INACTIVATION
FORMALDEHYDE REDUCTION and
CONCENTRATION (cut-off 30.000)
STERILE FILTRATION
PREADSORPTION

STORAGE
⇓
FORMULATION
⇓
FILLING

**Figure2a**

**PROJECT A+B/MPL / EXP 98299 : Serological analysis**

| | | | | | |
|---|---|---|---|---|---|
| Mice: | NMRI | Antigen: | HB, HA | Vehicule: | Alum |
| Route: | sc | | Immunostimulant: | MPL | |
| Immunisation scheme (day): | | 0,28 | | | |

| | | |
|---|---|---|
| 1 | HAB112B6 | HB20/ AlPO4 + HA720/ Al(OH)3 |
| 2 | DHAB713 | HB20/AlPO4 / MPL50 + HA1440/ Al(OH)3 |
| 3 | DHAB717 | HB20/ AlPO4 + MPL50/Al(OH)3 + HA720/ Al(OH)3 |
| 4 | DHAB718 | HB20/ AlPO4 + MPL50/AlPO4 + HA720/ Al(OH)3 |
| 5 | DHAB716 | HB20/AlPO4 / MPL50 + HA720/ Al(OH)3 |

**1) Response on pool on 28 days post I**

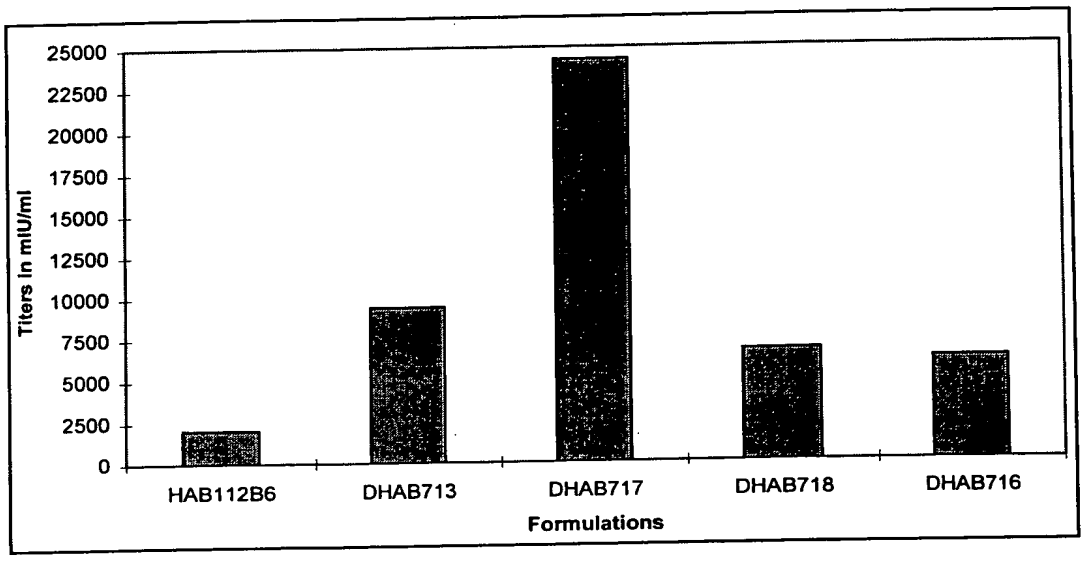
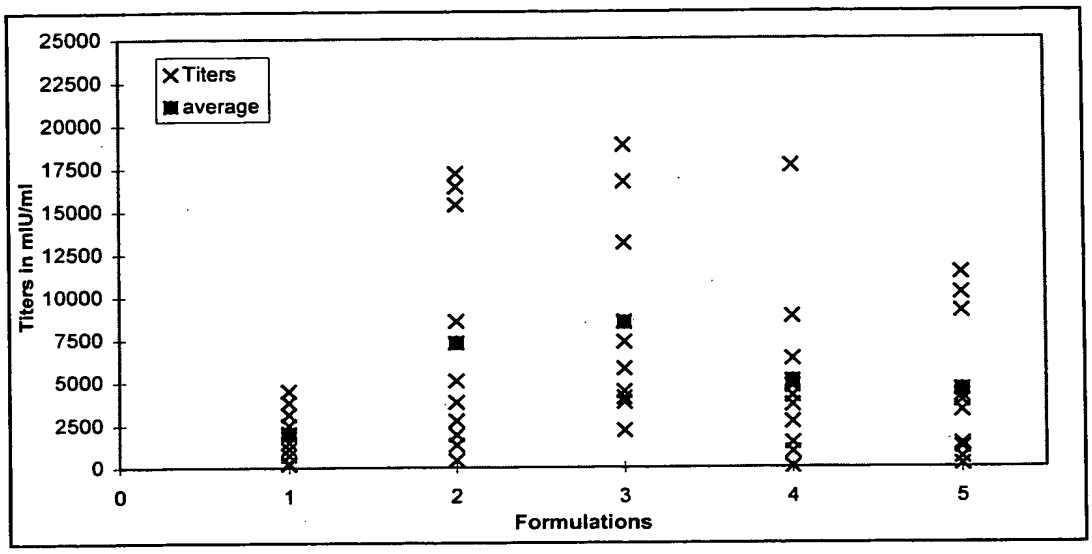


**Figure 2b**

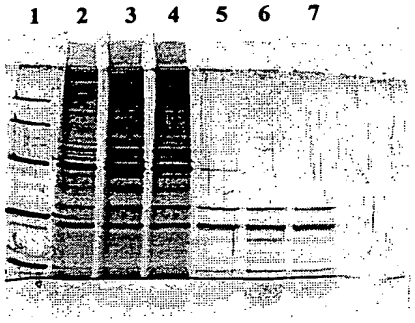
**2) Response on individuals on 28 days post I**



## Figure3

**SDS PAGE f HAV pool before utilising Andre et al PROCESS) & after the MMP pr cess f example 1b (HAV-MMP) Early trypsin single permeation step pr cess.**
Proteins are fractionated on 12.5% SDS-PAGE. They are visualized by silver staining

Lane 1: Low MW standards (14.4,21.5,31.0,45.0,66.2,97.4 kDa)
Lane 2: Andre et al (lot 5199)
Lane 3: Andre et al (lot 5200)
Lane 4: Andre et al (lot 5201)
Lane 5: HAV- MMP (lot 7199)
Lane 6: HAV-MMP (lot 7200)
Lane 7: HAV-MMP (lot 7201)

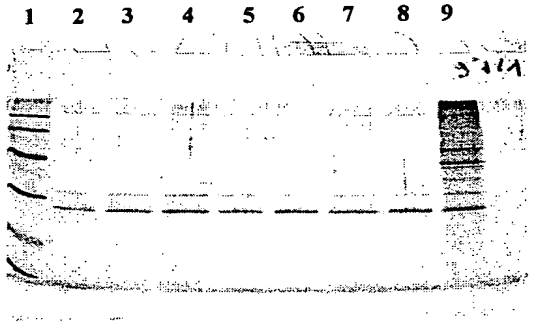



Note: lot numbers refer to distinct virus pools

## Figure 4

**SDS PAGE of HAV pool before (andre et al) & after the processof example 1a with twostep permetion chromatography (HAV-MMP)**
Proteins are fractionated on 12.5% SDS-PAGE. They are visualized by silver staining

Lane 1: Low MW standards (14.4,21.5,31.0,45.0,66.2,97.4 kDa)
Lanes 2 to 8: HAV-MMP (lots 6029 to 6032 and 6034 to 6036
Lane 9: Anre et al (lot 5010)




Note: - lot numbers refer to distinct virus pools
-Scan of a gel photograph